

# Auxin Responsiveness of a Novel Cytochrome P450 in Rice Coleoptiles<sup>1</sup>

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An early auxin-induced gene was isolated from rice (*Oryza sativa* L. subsp. *japonica* cv Nihonmasari) coleoptiles by a fluorescent-labeled differential display screen. The full-length gene contains conserved domains characteristic for the cytochrome P450 superfamily. This gene, designated as *CYP87A3*, was weakly expressed in dark-grown coleoptiles but was up-regulated rapidly and transiently when coleoptile segments were incubated in 5  $\mu$ M indole-3-acetic acid. This induction by auxin could not be suppressed by cycloheximide. Depletion of segments from endogenous auxin reduced the amount of *CYP87A3* transcripts. The *CYP87A3* transcript level was rapidly, although transiently, up-regulated in response to light as well. The observed pattern of gene regulation might indicate a role in the suppression of auxin-induced coleoptile growth. The role of *CYP87A3* is discussed with respect to auxin signaling in the regulation of coleoptile growth.

Coleoptiles represent a classical model system to study the control of growth-related signaling, because they grow exclusively by cell elongation (Wada, 1961) and respond to environmental signals. The plant hormone auxin plays a central role in the control of coleoptile elongation. As a matter of fact, auxin was discovered as a powerful growth substance that is produced mainly in the coleoptile tip, migrates basipetally, and is crucial for the signal-dependent regulation of growth (Went, 1928).

Although the importance of basipetal auxin transport has been confirmed by a wealth of data collected over several decades (for review, see Masuda et al., 1998), the role of auxin in the signal-dependent growth response appears to be complex. As shown by recent data, auxin distribution depends on several intrinsic activities, mainly synthesis, conjugation/deconjugation, and catabolism (for review, see Normanly, 1997). Moreover, changes in auxin transport between neighboring tissues and/or local concentrations can influence growth. For instance, a correlation between growth inhibition and a decrease in the level of extractable indole-3-acetic acid (IAA) could be demonstrated in the epidermis of irradiated pea (*Pisum sativum*) seedlings (Behringer and Davies, 1992). In addition, sensitivity/responsiveness of the target

tissue to auxin have to be considered (Trewavas, 1981). Because of this complexity, the events underlying signaling mediating auxin-triggered cell elongation are still far from being understood.

Growing evidence has appeared concerning regulation of auxin action at the level of gene expression (for example, expressional control of primary auxin-responsive genes, such as members of GH3 or Aux/IAA family; see Leyser, 2002; Hagen and Guilfoyle, 2002; Nakazawa et al., 2001). From the expression pattern of such genes, their role in signaling as well as their putative function have been inferred. We therefore ventured to isolate auxin-regulated gene(s) related to auxin-induced elongation. Using fluorescent differential display (FDD; Kuno et al., 2000), we screened for a subset of auxin-induced genes, whose expression was correlated with the auxin-induced elongation. This was accomplished by including a rice (*Oryza sativa*) mutant line, *Yin-Yang*, with an elevated auxin responsiveness of growth (Wang and Nick, 1998) as a reference system for the differential display screen. We identified several auxin-inducible cDNA fragments that were differentially regulated between wild type (WT) and mutant (Waller et al., 2002). One of these, *CYP87A3*, a new member of the cytochrome P450 (CYP) superfamily, was induced by auxin in the WT, but not in the mutant. In addition, this gene was light-responsive.

The CYP proteins are encoded by a highly divergent gene superfamily, present in both pro- and eukaryotes. Classic CYP proteins are heme-containing monooxygenases that catalyze numerous enzymatic reactions involved in detoxification as well as in biosynthetic pathways. In plants, they have been shown to participate in the synthesis of hormones, lignin intermediates, sterols, terpenes, and flavonoids (for review, see Schuler, 1996). Some CYP proteins have been shown to be important regulators during plant

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growth and development (Szekeres et al., 1996; Choe et al., 1998; Kim et al., 1998; Zhao et al., 2002).

To characterize this putative negative regulator of the auxin response, we cloned the full-length cDNA of *CYP87A3* and analyzed the expression pattern in coleoptiles under various treatments. We report here that *CYP87A3* is a primary auxin response gene. It is transiently up-regulated by auxin (accompanied by a stimulation of growth) but also by irradiation (accompanied by an inhibition of growth). We therefore discuss the highly dynamic *CYP87A3* expression and possible mechanisms of its regulation in the context of auxin-mediated growth regulation.

## RESULTS

### *CYP87A3* Was Isolated from a FDD Screen for Auxin-Induced Transcripts

Using fluorescence-labeled differential display, auxin-induced transcripts were compared between WT rice (*O. sativa* L. subsp. *japonica* cv Nihonmasari) and the *Yin-Yang* mutant, where the growth response to auxin is increased by about 50% (Wang and Nick, 1998). Excised coleoptiles of 6-d-old WT and *Yin-Yang* plants were incubated for 1 h in water to deplete them from endogenous auxin and then incubated for 1 h in 5  $\mu\text{M}$  IAA (Fig. 1A), the IAA concentration optimal for growth induction (Waller

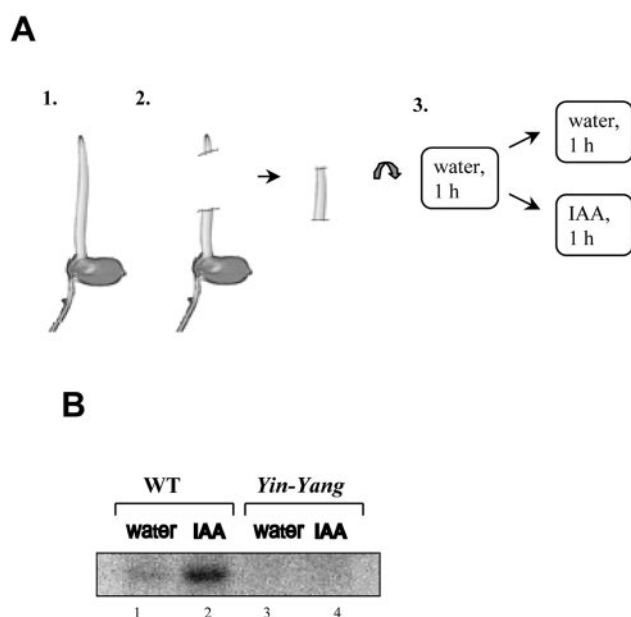
et al., 2002). As a negative control, coleoptiles were kept in water for 2 h. FDD analysis of total RNA from each treatment showed that only a few auxin-induced genes were differentially expressed in WT and *Yin-Yang* (Waller et al., 2002). The expression of one of them, here and further referred to as *CYP87A3*, was up-regulated by auxin in the WT, whereas it was virtually absent in the *Yin-Yang* mutant (Fig. 1B). We therefore focused on two main objectives: to clone the complete gene, and to analyze the regulation of its expression with respect to auxin signaling.

### *CYP87A3* Is Coding for a Protein That Belongs to the CYP Superfamily

To obtain the full-length *CYP87A3* cDNA, the RACE approach was applied. The predicted length of the mRNA (2,185 bp; accession no. AJ459255) was consistent with the observed size estimated from northern-blot analysis. The TATA box (TATATAA) is located 33 bp upstream of the transcription start. By searching the Monsanto rice sequencing database (<http://www.rice-research.org>) we found that the *CYP87A3* genomic sequence was localized in the rice clone OSM1165. The *CYP87A3* gene contains nine exons and eight introns and is 4.5 kb long excluding the promoter region. The *CYP87A3* genomic sequence is now available as part of the sequence of bacterial artificial chromosome clone OSJNBA0088122 (accession no. AL607001) and is located on chromosome 4.

Two reading-frame translations of the *CYP87A3* cDNA yielded numerous stop codons throughout the whole length, whereas the third frame revealed an open reading frame of 1,542 bp coding for a deduced protein of 514 amino acids. The estimated molecular mass of this protein is 57.5 kD, and the predicted pI is 9.0. The putative *CYP87A3* protein showed homology to members of the CYP superfamily.

By definition, CYP sequences are classified into the same family (designated by the first number) when they are >40% identical to one another at the level of the amino acid sequence, those belonging to the same subfamily (designated by the letter) are >55% identical, and those corresponding to alleles of the same locus are >97% identical (Schuler, 1996). BLAST searching indicated that *CYP87A3* protein shows substantial amino acid identity (61%) with a predicted protein of Arabidopsis (chromosome 1; accession no. NP-172734) designated *CYP87A2* and 55% amino acid identity with putative *CYP87A1* from sunflower (*Helianthus annuus*; accession no. AF216313, partial mRNA sequence). Therefore, the identified gene was designated as a member of the rice *CYP87A* subfamily and named *CYP87A3*. The function of both of the closest homologs is unknown. The closest homologs of known function are constitutive photomorphogenesis and dwarfism (CPD; 39% amino acid identity; accession no. Q42569), DWF4 (steroid 22-hydroxylase; accession no. AF044216.1), and ROTUNDIFOLIA3



**Figure 1.** Fluorescence differential display screen for auxin-inducible transcripts. A, Scheme of the experiment: 1, coleoptiles were grown for 6 d in darkness; 2, coleoptile segments were excised; and 3, segments were predepleted in water for 1 h and then incubated for 1 more h in IAA or in water. B, A part of an FDD gel image showing the *CYP87A3* expression pattern in WT and the mutant *Yin-Yang*. Total RNA was isolated from coleoptile segments incubated for 1 h either in distilled water (lanes 1 and 3) or in 5  $\mu\text{M}$  IAA (lanes 2 and 4).

(34% amino acid identity; accession no. Q9M066). They belong to the A, B, and C subfamilies of the CYP90 family from Arabidopsis. Similar levels of homology were found for the tomato (*Lycopersicon esculentum*) DWARF protein CYP85 (38% amino acid identity; accession no. Q43147) and the maize (*Zea mays*) DWARF3, CYP88A1 (32% amino acid identity; accession no. Q43246). By database searching (Dr. Nelson WebPage [http://drnelson.utm.edu/rice.html]), we identified the two closest homologs of the CYP87A3 in rice, sharing the same locus on chromosome 4 (OSJN00128): One putative protein derived from this locus was 57% identical and thus could be assigned to the same subfamily, CYP87A; a second putative protein from this locus had 48% of amino acid identity to CYP87A3.

CYPs are heme-containing proteins that primarily catalyze reactions of monooxygenation/hydroxylation (Werck-Reichhart et al., 2000). The catalytic domain containing a ferriprotoporphyrin IX (heme) prosthetic group covalently attached to a Cys is characterized by a highly conserved, C-terminal F-G-R-C-G motif (Schuler, 1996), which is also found in CYP87A3 (Fig. 2). An amino acid alignment of two CYP proteins involved in brassinosteroid biosynthesis (22 $\alpha$ -hydroxylase DWF4/CYP90B1 [Choe et al., 1998]; 23 $\alpha$ -hydroxylase CPD/CYP90A1 [Szekeres et al., 1996]) and other CYP proteins that are involved in gibberellin biosynthesis and dwarfism (DWF3/CYP88A1 [Winkler and Helentjaris, 1995]; ROTUNDIFOLIA/CYP90C1 [Kim et al., 1998]; and DWARF/CYP85 [Bishop et al., 1996]) with CYP87A3 revealed highly conserved domains in these proteins in addition to the C-terminal heme-binding domain. All of these proteins exhibit a central region with steroid substrate-binding and oxygen-binding domains (Fig. 2). An N-terminal transmembrane helix can be predicted with high probability from a hydrophobicity analysis of the CYP87A3 sequence. An N-terminal endoplasmic reticulum anchor domain that is not cleaved off during posttranslational modification (Omura, 1999) is typical for most plant and many animal CYPs. A Pro-rich region (Fig. 2) downstream of the signal sequence that is highly conserved among classic CYP proteins can be identified in CYP87A3 as well (Schuler, 1996).

#### Features of Promoter and mRNA 3'-Untranslated Regions (UTRs)

Several putative auxin-responsive elements were found in the so far cloned 1-kb fragment of the CYP87A3 promoter using the Web Signal Scan Program and the PLACE database: (a) A GTTCCCAT motif, which differs in only one nucleotide from the (G/T) GTCCCAT found in promoters of several auxin-regulated genes such as the 165-bp fragment of pea *PS-IAA4/5* promoter, reported to contain an auxin-responsive cis-element (Guilfoyle et al., 1998).

(b) A TGACGTAA sequence, highly homologous to the TGA box of the E1 motif in the *GH3* promoter (Liu et al., 1994). (c) Sequences highly homologous to the A1 module of the *SAUR15A* promoter (Xu et al., 1997). In addition, the CYP87A3 promoter contains numerous short sequences highly homologous to the auxin-responsive *ocs*-like element TGATGTAAGA-GATTACGTAA of the *GH2/4* promoter (Abel and Theologis, 1996). *Ocs*-like elements are activated not only by auxin, but also by other growth substances and stress-inducing agents (Abel and Theologis, 1996). It is interesting that in the CYP87A3 promoter, the *ocs* core sequences overlap with the TGA box or are adjacent to other putative auxin-responsive elements.

The cis-elements potentially involved in light-regulated transcription appeared to be most abundant in the 5' region of CYP87A3. In addition to classic motifs for light regulation such as the GATA-box, the GT1-consensus, and the I-box (Donald and Cashmore, 1990; Gilmartin et al., 1990; Terzaghi and Cashmore, 1995; Rose et al., 1999; Zhou, 1999), further cis-elements characteristic for light-regulated genes in different plants are present in the CYP87A3 promoter. It also contained binding sites for embryo- and root-specific expression.

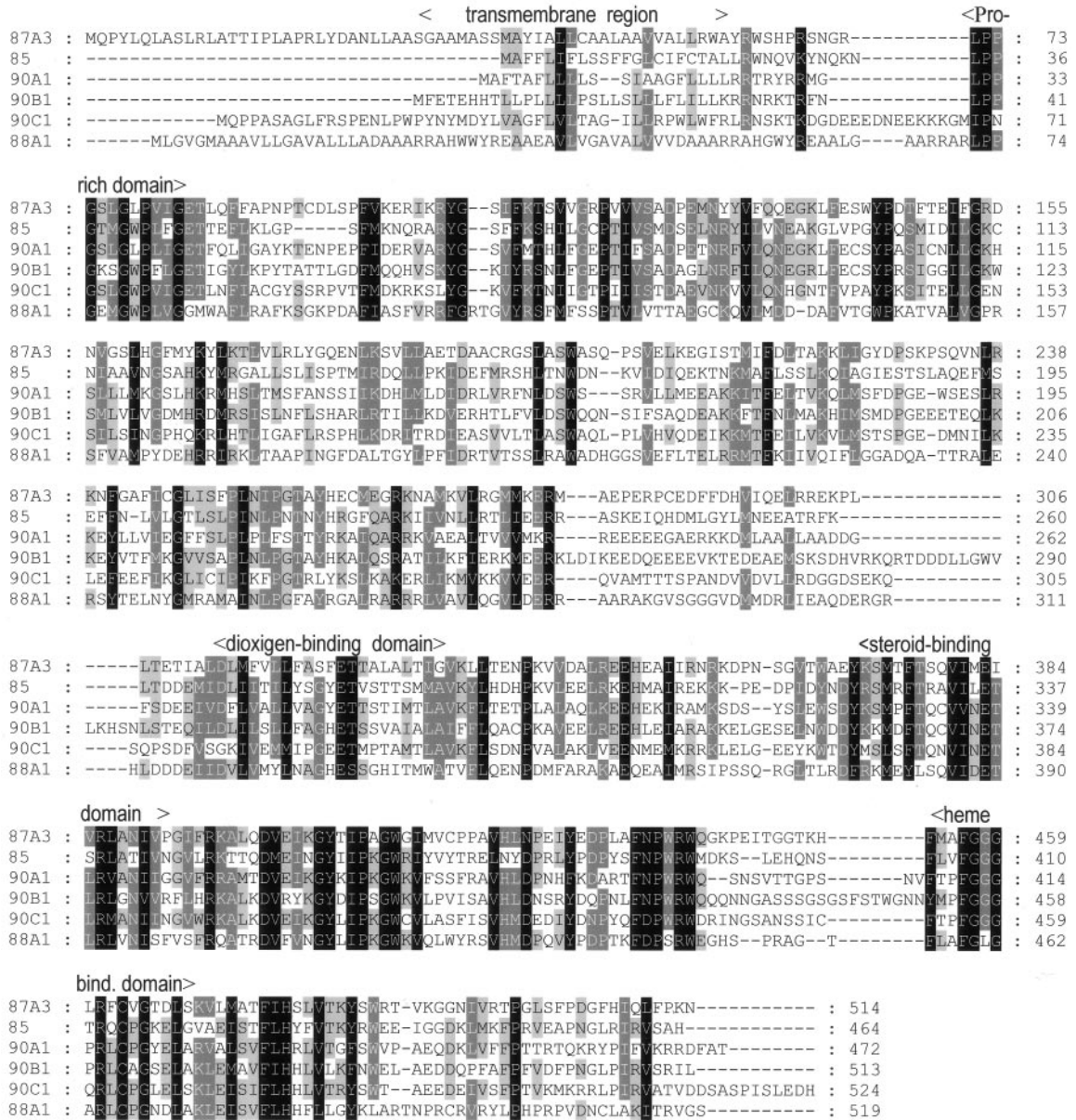
The Plant CARE promoter analysis program revealed additional binding sites for trans-factors involved in jasmonate (TGACG-motif), gibberellin (TATC-box and P-box, CCTT), ethylene (ERE, ATTT), and wounding (WUN-motif, (C)AATT) responsiveness. Our preliminary findings indicate that CYP87A3 expression is influenced by ethylene and jasmonic acid, whereas wounding had virtually no effect (data not shown).

A comparison of the CYP87A3 3'-UTR with the consensus sequence from the so-called downstream element (DST) of SAUR genes, which was shown to be responsible for rapid mRNA degradation (McClure and Guilfoyle, 1989; Gil and Green, 1996), revealed several highly homologous motifs. These multiple motifs corresponded to individual components of the DST element, but they were arranged in a different order. Although the 3'-UTR is AT-rich, its sequence did not show the multiple overlapping ATTTA motifs that are thought to be instability determinants in mammalian cells (Greenberg and Belasco, 1993).

#### Localization of CYP87A3

To determine the subcellular localization of the CYP87A3 protein, dark-grown rice seedlings were transiently transformed with CYP87A3:cyano-green fluorescent protein (CFP) fusion constructs using a biolistic method. Both cells with strong and weak expression were examined. Whereas in cells transformed with the control construct 35S:CFP the CFP was distributed homogeneously throughout the cell





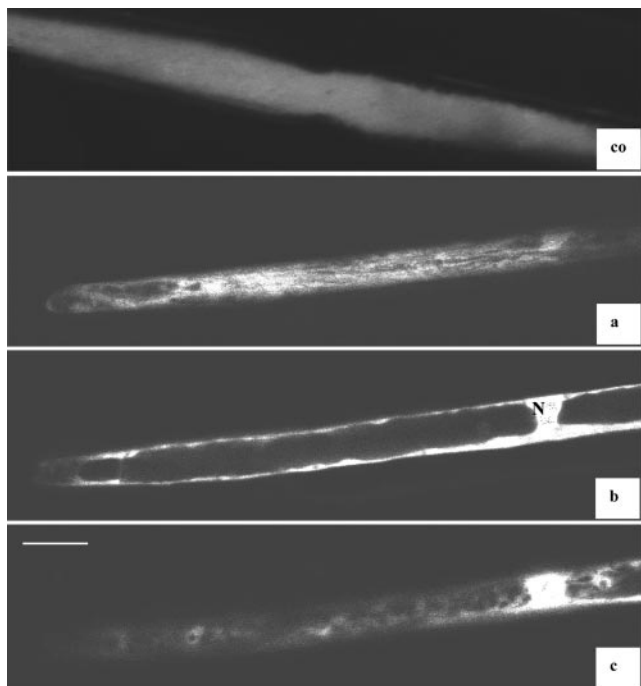
**Figure 2.** Multiple sequence alignment of CYP87A3 with related CYP proteins. CYP87A3 exhibits homology to the 23 $\alpha$ -hydroxylase CPD (CYP90A1), the 22 $\alpha$ -hydroxylase DWF4 (CYP90B1), ROTUNDIFOLIA (CYP90C1), DWF3 (CYP88A1), and DWARF (CYP85). The locations of the conserved domains are indicated above the sequences. Amino acid residues that are identical between the compared sequences are indicated by reverse font; 80% conserved, by white letters in gray boxes; 60% conserved, by gray boxes.

(Fig. 3, co), a specific pattern of CFP distribution was observed for the CYP87A3:CFP fusion protein (Fig. 3, a-c). In addition to a signal around the nucleus, strong expression was noted in broad longitudinal strands adjacent to the outer cell surface and as a characteristic signal surrounding unstained vesicular structures (“negative staining”) in the cytoplasm. In cells with weaker expression, only these “negatively stained” vesicular structures were observed in the cell cortex. However, a strong nuclear signal (distributed throughout the nucleus) was present irrespective of the expression level.

Northern-blot analyses of different organs—primary leaves, distal and basal parts of young leaves, old leaves, and roots—that had been incubated either in water or in auxin indicate that CYP87A3 is only expressed in roots and coleoptiles, but not in leaves (data not shown).

**Time Course and Dose Response of Auxin Induction**

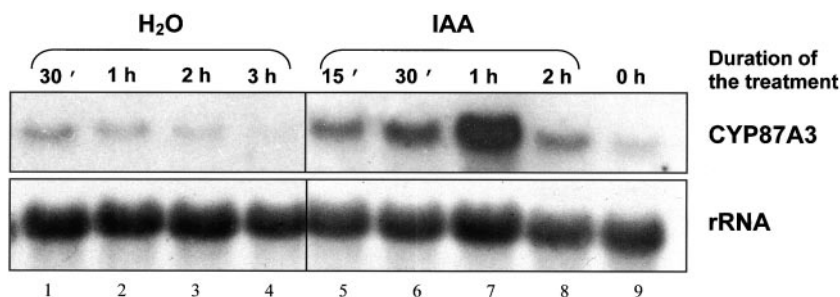
For a kinetic study, RNA was isolated from coleoptile segments of the WT that had been incubated in



**Figure 3.** Subcellular localization of 35S:CYP87A3:CFP fusion protein. The transient expression of CYP87A3 with in-frame C-terminally fused CFP was analyzed in coleoptile epidermal cell by confocal laser scanning microscope. co, Control, 35S::CFP; a through c, sequential confocal sections through the same cell expressing CYP87A3::CFP, beginning from the outer cell surface. White bar = 20  $\mu\text{m}$ ; N, nucleus.

IAA or water for different time intervals. Expression of *CYP87A3* was low in freshly excised, dark-grown coleoptiles (Fig. 4, lane 9). When endogenous auxin was depleted by incubating the segments in water, the signal decreased gradually with longer incubation periods (Fig. 4, lanes 2–4). However, for short time intervals of the incubation of segments in water (30 min), the signal was slightly stronger compared with directly harvested coleoptiles (Fig. 4, compare lanes 1 and 9). When the coleoptile segments were incubated in 5  $\mu\text{M}$  IAA, the *CYP87A3* was strongly but transiently induced with a lag time of less than 15 min and maximal expression at 1 h. The mRNA level declined down to the initial level within the 2nd h after the addition of auxin (lanes 5–8).

**Figure 4.** Kinetics of *CYP87A3* expression in response to exogenous IAA. Total RNA (15  $\mu\text{g}$  per lane) was isolated from coleoptile segments that either had been excised directly from seedlings grown for 6 d in darkness (lane 9) or that after excision had been incubated in water (lanes 1–4) or in 5  $\mu\text{M}$  IAA (lanes 5–8), respectively. The same membrane was hybridized with probes specific to *CYP87A3* and 28S rRNA (loading control).



The IAA dose response curve for *CYP87A3* expression revealed already for 0.005  $\mu\text{M}$  IAA a slight induction as compared with the water control (Fig. 5, compare lanes 1 and 2). Between 0.005 and 5  $\mu\text{M}$  IAA, the transcript was progressively enhanced with a maximal plateau at 0.5 to 5  $\mu\text{M}$  (Fig. 5, lanes 4 and 5, respectively). When auxin concentration was raised further, however, the signal declined again to almost the uninduced level (Fig. 5, lanes 6 and 7).

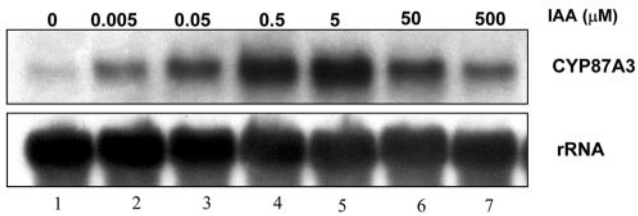
### CYP87A3 Is Strongly Induced by Cycloheximide

Cycloheximide, an inhibitor of protein synthesis, has been often used in investigations of genes involved in early steps of auxin signaling (for review, see Abel and Theologis, 1996). A wide range of genes, termed primary auxin-responsive genes, is induced within minutes after auxin application, and this induction is not suppressed by inhibition of protein translation, indicating that all proteins necessary to initiate transcription of primary auxin-responsive genes are already present in the cell and can be activated by auxin.

*CYP87A3* transcript level was already greatly induced by 30 min of incubation in cycloheximide (Fig. 6, lane 3). During the following hour of cycloheximide treatment, the signal became even stronger (lane 6). Interestingly, cycloheximide alone stimulated the *CYP87A3* transcript to a greater extent than IAA alone (Fig. 6, compare lanes 2, 3, 5, and 6). The combination of cycloheximide and IAA yielded a slight superinduction as compared with the signal obtained for cycloheximide alone (Fig. 6, lane 7; for better resolution, shorter film exposure time is shown in the middle row of the figure).

### Light Affects CYP87A3 Expression

Because the FDD screen showed that light modulates the expression of *CYP87A3*, we followed the abundance of the transcript over time for two different intensities of red light. For the low intensity (3.8  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), the transcript remained stable or even slightly increased up to 1 h of irradiation and then disappeared during the 2nd h of irradiation (Fig. 7A). For the high intensity (38.9  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), the initial increase was more rapid (with a peak at 30



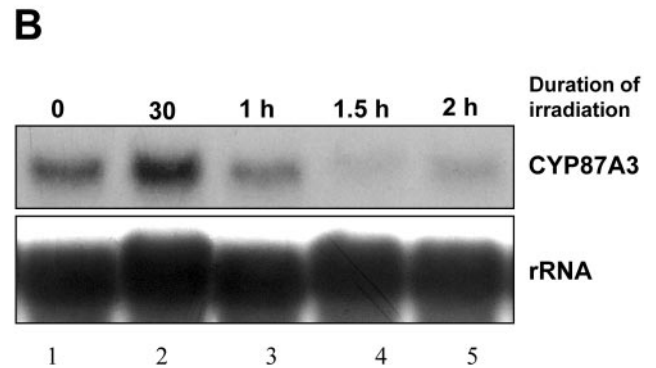
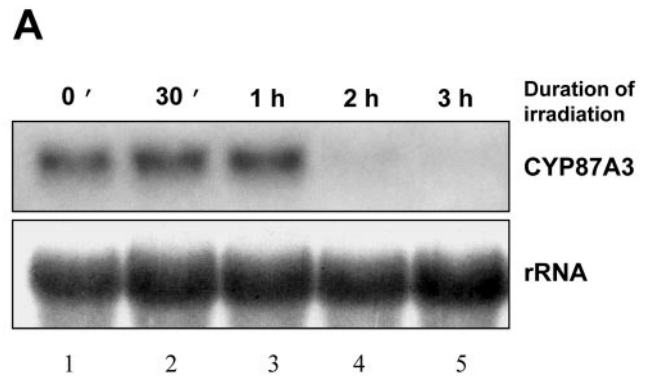
**Figure 5.** The response of CYP87A3 transcripts to different concentrations of IAA. Total RNA (15 μg per lane) was isolated from coleoptile segments that had been incubated for 1 h in IAA of different concentrations directly after excision. Top row, CYP87A3-specific probe was used; bottom row, loading control shown by hybridization with 28S rRNA-specific probe.

min) and much more pronounced in amplitude. Again, the transcript disappeared during the hour following the peak (Fig. 7B). A similar effect was observed during irradiation of seedlings with blue and far-red light (data not shown).

**DISCUSSION**

**CYP: From Regulation to Function**

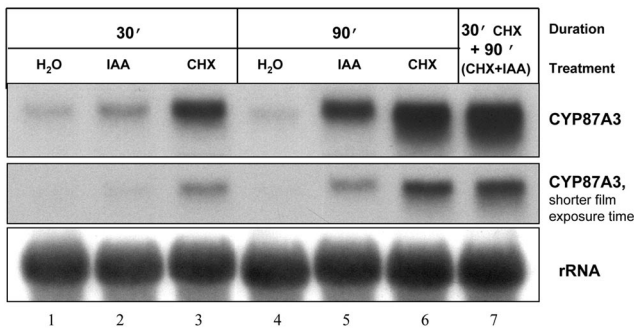
CYP87A3, a putative member of the rice CYP monooxygenase superfamily, was isolated in a FDD screen for auxin-inducible genes related to the auxin responsiveness of growth. CYPs are involved in oxidative metabolism of different endogenous and exogenous lipophilic substrates (Mizutani et al., 1998). The catalyzed reaction is usually a mono-oxygenation, with the insertion of one oxygen atom (as a hydroxyl group) into a substrate and formation of one water molecule, but additional more atypical activities, such as dimerizations, isomerizations, dehydrations, and reductions have also been reported (for review, see Halkier, 1996; Werck-Reichhart et al., 2000). In the generalized reaction scheme, electrons are trans-



**Figure 7.** CYP87A3 transcripts are transiently induced by red light. Total RNA (14 μg per lane) was isolated from coleoptiles after irradiation for different time intervals. A, Weak red light (RL), 3.8 μmol m<sup>-2</sup> s<sup>-1</sup>. B, Strong red light, 38.9 μmol m<sup>-2</sup> s<sup>-1</sup>. The irradiation time is indicated. For details refer to the legend of Figure 4.

ferred from NADPH to the CYP via FAD and FMN flavoproteins called CYP reductases. Plant CYPs are usually bound via their N terminus to the cytoplasmic surface of the endoplasmic reticulum. They have a highly hydrophobic N-terminal “signal anchor sequence,” consisting of about 25 amino acid residues, which targets and anchors the CYP molecules to the membrane (for review, see Omura, 1999). By hydrophathy analysis, this plasma membrane anchor sequence was predicted to be present in CYP87A3 as well. The analysis of subcellular localization of CYP87A3 by means of transient expression of its fusion with a fluorescent protein revealed a highly specific distribution in the cell cortex of transformed epidermal cells consistent with a targeting to the endomembrane system in these highly vacuolated cells. In addition, a part of the signal was found within the nucleus. This might be caused by the overexpression of the fusion protein. As a consequence, the putative binding partner of the protein would be saturated so that the free fusion protein would become dislocalized.

Due to the diversity of pathways requiring monooxygenase activity, the expression of CYPs has been reported to be modulated by a wide variety of envi-



**Figure 6.** CYP87A3 transcripts are elevated after cycloheximide treatment. Total RNA (15 μg per lane) was isolated from coleoptiles that have been treated with cycloheximide and/or auxin. When coleoptiles were treated by cycloheximide in combination with auxin (lane 7), cycloheximide was applied 30 min before IAA to ensure inhibition of protein synthesis before the exogenous auxin could enter the cell. The working concentrations were 5 μM for IAA and 70 μM for cycloheximide. For details, refer to the legend of Figure 4. CHX, cycloheximide.



ronmental factors such as light, fungal elicitors, and wounding, as well as by developmental signals (Dixon and Paiva, 1995; Mizutani et al., 1998). In all cases, the induction profile was found to be specific for a particular CYP or subset of CYPs and not the result of a global induction of CYP genes. Analyzing the response pattern of seven CYP transcripts after treatment of maize seedlings with chemical inducers, Persans et al. (2001) have found that each of the transcripts had distinct developmental, tissue-specific, and chemical cues regulating their expression even when they encode CYPs within the same biosynthetic pathway. These observations were consistent with results obtained from analyses on the transcriptional regulation of other CYPs, for example CYP72A2 (induced by wounding and cytokinin and involved in the defense responses of tobacco [*Nicotiana tabacum*]; Mujer and Smigocki, 2001), CPD (suppressed by brassinosteroids and participating in brassinolide biosynthesis; Mathur et al., 1998), or CYP83B1 (auxin inducible, containing AuxREs in the promoter, and involved in the biosynthesis of indole glucosinolates from Trp, a branching point between the biosynthesis of IAA and indole glucosinolates; Bak et al., 2001).

These findings demonstrate that the pattern of CYP regulation is strictly correlated to their biological function. Following this line of argument, the CYP87A3 gene product might be placed in the tuning of growth responses. This is supported by the FDD screen that led to the isolation of this gene, where CYP87A3 transcripts were almost undetectable in coleoptiles of the mutant *Yin-Yang* that are characterized by an elevated auxin responsiveness of growth, but were auxin-inducible in the WT. A similar reduction of the CYP87A3 transcript level was found for further mutant, *hebiba*, with elevated auxin responsiveness of growth (Riemann et al., 1993). The CYP87A3 protein is therefore most likely a negative regulator for the auxin responsiveness of growth. The transient up-regulation of this gene in irradiated coleoptiles is accompanied by a substantial decrease of endogenous auxin (Riemann et al., 1993), which might be responsible for the decrease in growth. Thus the transient induction of CYP87A3 by light might indicate an increased responsiveness to auxin. Whether light modifies the auxin responsiveness of CYP87A3 at the level of transcription or of RNA stability is still undetermined. Alternatively, irradiation might lead to auxin-independent gene regulation. In both cases, however, it may control the auxin response of downstream targets of the CYP87A3 protein.

In this context it is possible, for instance, that the CYP87A3 gene product might be directly involved in IAA de-activation/transport inhibition or, alternatively, in the biosynthesis of auxin antagonists. An attractive, but still speculative target might be, for example, flavonoids that have been discussed for

several decades to be involved in regulation of auxin transport (Stenlid, 1976; Brown et al., 2001, and refs. therein). Certain flavonoids were shown also to play role in auxin breakdown in plants (Furuya et al., 1962; Stenlid, 1963; Rao, 1990 and refs. therein). For instance, Mathesius (2001) found that regulation of auxin breakdown during nodule organogenesis is under control of flavonoids in white clover (*Trifolium repens*). Taking into account that CYPs are prominent enzymes in flavonoid biosynthesis (Schuler, 1996), one could speculate about a parallel in rice coleoptiles: Differential CYP87A3 expression might contribute to local distributions of specific flavonoids. Because flavonoids seemed to participate in regulation of auxin transport/oxidation, CYP87A3 could be involved in the control of local auxin levels. Therefore, we plan to express recombinant functional CYP87A3 to test its effect on IAA oxidation and/or to screen for its potential substrate(s).

#### Auxin Regulates CYP87A3 Expression

We have found that auxin application rapidly induced CYP87A3 expression. Such a rapid response that is not inhibited by cycloheximide is characteristic for early or primary auxin response genes. We observed a strong induction of CYP87A3 in coleoptile segments that were treated with cycloheximide alone. The combination of cycloheximide with IAA seemed to have very little additional effect on this induction. A similar cycloheximide effect was shown for several other early auxin response genes, including members of the SAUR and Aux/IAA families, which can be induced by cycloheximide alone (McClure et al., 1989; Abel and Theologis, 1996). On the basis of a detailed study of the effect of protein synthesis inhibition on the expression of *Ps-IAA4/5* and *Ps-IAA6*, Koshiba et al. (1995) proposed a dual effect of cycloheximide by (a) activation of transcription and (b) stabilization of the inducible mRNAs. To explain the first effect, it has been proposed that cycloheximide prevents synthesis or activation of a short-lived transcriptional repressor whose subsequent degradation or deactivation results in the activation of auxin-inducible genes (Koshiba et al., 1995). In fact, Aux/IAA proteins have been shown to suppress the expression of reporter genes containing highly active auxin-responsive elements (Ulmasov et al., 1997) and are therefore good candidates for such a repressor. The second effect has been explained by the disruption of translation-coupled mRNA degradation or by the depletion of the pool of a labile enzyme that degrades specific transcripts (Koshiba et al., 1995). The analysis of the 3'-UTR of CYP87A3 revealed several sequences that are identical to motifs in the DST instability sequence. This means that CYP87A3 expression might be controlled at the level of sequence-specific mRNA degradation as well. To distinguish between the particular mechanisms re-

sponsible for the superinduction of *CYP87A3* by cycloheximide, further functional analysis of the UTRs would be desirable.

The regulation pattern of *CYP87A3* transcripts contains two remarkable features:

(a) *CYP87A3* transcripts are only transiently (peak at 1 h) increased in response to auxin. After 2 h of auxin treatment, the transcript level had almost returned to the original situation. In contrast, the transcripts of another primary auxin response gene, *OsARF1*, remain elevated for several hours after the addition of auxin (Waller et al., 2002), which is typical for other classical primary auxin response genes such as members of the Aux/IAA family (at least up to 5 h; Theologis et al., 1985), *GH3* (Hagen and Guilfoyle, 1985), or the *SAUR* family (McClure and Guilfoyle, 1987; McClure et al., 1989; Gee et al., 1991). Transient induction is usually characteristic for regulatory components involved in signaling pathways. Assuming that *CYP87A3* codes for such a regulatory component involved in tissue adaptation to higher auxin concentration, a rapid return to the initial lower transcript level would restore the cellular competence for subsequent responses to changing auxin concentration.

(b) The dose response curve, particularly its response to higher auxin concentrations differs from classic primary auxin response genes such as *GH3* and *SAUR*. Reaching a maximum plateau at concentration 0.5 to 5  $\mu\text{M}$ , the induction of *CYP87A3* sharply decreases in amplitude when IAA concentrations are raised above 5  $\mu\text{M}$ . This bell-shaped curve parallels the dose response curve of auxin-induced growth (Waller et al., 2002). Interestingly, a similar dose response was shown for *OsARF1* with a maximum response to 1 to 3  $\mu\text{M}$  IAA (Waller et al., 2002) as well as for members of Arabidopsis Aux/IAA family (optimum response at 10  $\mu\text{M}$  IAA; Abel et al., 1995). In contrast, the transcription of *GH3* or *SAUR* genes was reported to persist even very high concentrations of auxin (100  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid in the case of *GH3* [Hagen and Guilfoyle, 1985]; up to 10 mM 2,4-dichlorophenoxyacetic acid in the case of *SAUR* [McClure and Guilfoyle, 1987]).

Currently, we are performing experiments to determine possible coregulators of *CYP87A3* expression. Our further research will focus on a functional analysis of the promoter region as well as on getting insight into the cross-talk between light signaling and auxin-induced elongation growth.

## MATERIALS AND METHODS

### Plant Material

Japonica rice (*Oryza sativa* L. subsp. *japonica* cv Nihonmasari) was used for the experiments. For FDD analyses, seedlings of the mutant *Yin-Yang* (Wang and Nick, 1998) were used in addition to the WT. The seedlings were grown on floating meshes as described by Nick et al. (1994) in photobiological darkness (using black boxes wrapped in black cloth) and kept in dark chambers at 25°C. Six days after germination, the apical 2 to 3 mm of the

coleoptile was removed, and subapical segments of 10 to 15 mm length were used for further experiments (Waller et al., 2002). The segments were incubated in water or in different concentrations of IAA (Fluka, Buchs, Switzerland) under continuous rotation in a top-over shaker. If not stated otherwise, all preparations were made in green safelight. For the light experiments, the seedlings were irradiated for different time intervals. The light sources for red light ( $\lambda_{\text{max}}$  660 nm, 3.8 and 38.9  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ), far red light ( $\lambda_{\text{max}}$  730 nm, 15.9  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ), blue light ( $\lambda_{\text{max}}$  430 nm, 22.8  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ) and green safelight ( $\lambda_{\text{max}}$  550 nm) are described in detail by Mohr et al. (1964). All light measurements were performed using a Tektronix-J16 photoradiometer (Tektronix, Beaverton, OR).

For the transient transformation assay, 4-d-old seedlings were attached to microscopic slides with their caryopses using surgical adhesive in green safelight. After transformation, slides with attached coleoptiles were returned to the dark and placed in vertical position to allow the seedlings grow further. The seedlings were analyzed the next day.

### FDD

FDD was performed as described by Waller et al. (2002). First-strand synthesis was performed with 2.5  $\mu\text{g}$  of total RNA using a Texas Red-labeled 3'-anchored oligo(dT) primer (5'-Texas Red-T<sub>14</sub>G-3, Youkigouseikagaku, Tokyo) and the Superscript Preamplification System (Invitrogen, Carlsbad, CA). cDNAs were amplified by PCR using combinations of the Texas Red-labeled anchor primer and arbitrary 10-mer primers (Kit B, D, F, and X, Operon Technologies, Alameda, CA). Electrophoresis and detection of the PCR products were performed with an automated DNA sequencer (SQ5500, Hitachi).

### Cloning of cDNA

The cDNA of interest was isolated by preparative gel electrophoresis and excised from the gel as described by Kuno et al. (2000). The sequence of cDNA 3' end was obtained as described by Waller et al. (2002). For the isolation of the complete cDNA sequence of *CYP87A3*, reverse transcription PCR using gene-specific primers and the 5'-RACE System for Rapid Amplification of cDNA Ends (Invitrogen), FirstChoice RLM-RACE kit (Ambion, Austin, TX), and GeneRacer kit (Invitrogen) were used according to the manufacturers recommendations.

### RNA Isolation and Northern-Blot Analysis

Total RNA was isolated from rice coleoptiles either by phenol/chloroform extraction according to Ehmann et al. (1991) or by using the Rneasy Plant Mini kit (Qiagen, Hilden, Germany). Both methods resulted in comparable quality and quantity of isolated RNA. For all samples of a given northern blot, only one of the two methods was used.

Ten to 15  $\mu\text{g}$  of total RNA were separated by electrophoresis in 1% agarose-formaldehyde gels according to Davis et al. (1986) and transferred to Duralon UV membrane (Stratagene, La Jolla, CA). A [ $\alpha$ -<sup>32</sup>P]dCTP random-labeled probe corresponding to *CYP87A3* 3'-UTR was used. The DNA template for the probe was obtained with the following primer pair: 5'-GTGCAAAGATGTCGTTGGGTTTTT-3' and 5'-CACGGACTAGAGGT-AGCACTCGGT-3'.

The hybridization procedure and the probes for *OsARF1* detection were performed according to Waller et al. (2002). After washing with 2 $\times$  SSC, 1 $\times$  SSC, 0.5 $\times$  SSC, and 0.1 $\times$  SSC washing solutions (1 $\times$  SSC is 150 mM NaCl and 15 mM sodium citrate) containing 0.1% SDS at 63°C, membranes were autoradiographed for 1 to 4 d using an intensifying screen (Biomax, Eastman Kodak, Rochester, NY). To confirm equal transfer of the RNA to the membrane, the *CYP87A3/OsARF1* probe was removed after autoradiography, and the membrane was rehybridized with a probe corresponding to rice 28S rRNA.

### Quantitative Analysis of Northern-Blot Signals

For quantitative analyses, films were scanned and analyzed by ImageJ algorithm (<http://rsb.info.nih.gov/ij/index.html>). The actual RNA loading was calculated from analysis of 28S rRNA reprobated membranes. For every



treatment, three to seven repetitions were analyzed. All data were statistically processed.

## Transient Transformation of Rice Coleoptiles with CFP Constructs

The full-length CYP87A3 cDNA was obtained with the primer pair 5'-GTGATCTAGACATGCAGCCATATCTTCAGC-3' and 5'-GTTCTTAGG-GAAGAGCTGGATGAAAACC-3' and inserted in frame with a CFP using the vector (35S)<sub>2x</sub>-CFP/pUC kindly provided by K. Harter and F. Nagy (unpublished data). The CYP87A3 was C-terminally linked with CFP. The expression of the CFP fusion construct was driven by two copies of the 35S promoter. Transformation of bacteria and isolation of the plasmid DNA was performed using TOPO TA-Cloning kit (Invitrogen) and Qiaprep Spin Miniprep kit (Qiagen) according to the instructions of respective providers.

Gold particles (Sigma-Aldrich, St. Louis) were coated with the isolated plasmid DNA according to the following protocol: 25  $\mu$ L of a gold stock solution (60 mg mL<sup>-1</sup> in glycerol 50%), 5  $\mu$ L of plasmid DNA, 25  $\mu$ L of 2.5 M calcium chloride, and 10  $\mu$ L of 0.1 M spermidine solution were mixed by continuous vortexing and centrifuged for 1 min. The gold pellet was washed three times with 1 mL of 100%, 70%, and 100% ethanol. After a final centrifugation (1 min), the pellet was resuspended in 40  $\mu$ L of 100% ethanol. The whole resuspension was loaded onto particle bombardment grids. The bombardment was performed in a custom-made particle gun with a Helium pressure of 2 to 3 bar per shot and under vacuum conditions ( $\leq$ 0.8 bar). For transformation, etiolated 4-d-old rice seedlings glued onto a slide were used. The coleoptiles were analyzed by confocal laser scanning microscopy as described by Wang and Nick (2001) 24 h after transformation.

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